PHI-like immunoreactivity in the gallbladder and in vitro effect of porcine PHI on smooth muscle of the gallbladder

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Received 6 August 1984

The occurrence and distribution of PHI-like immunoreactivity in the guinea pig gallbladder has been analysed by radioimmunoassay and immunocytochemistry. Chromatography of gallbladder extracts by gel permeation and high-performance liquid chromatography revealed that guinea pig PHI-like immunoreactivity is of a similar size to that of porcine PHI but may differ in its amino acid sequence. Immunocytochemistry showed PHI-immunoreactivity to be localised to nerves found predominantly in the ganglionated plexus and the mucosal plexus of the gallbladder. Pure natural porcine PHI induced a dose-dependent relaxation of the isolated guinea pig gallbladder muscle which was not blocked by antagonists to acetylcholine, catecholamines, histamine, and 5-hydroxytryptamine. PHI may thus be one of the local factors involved in controlling gallbladder function.

PHI *VIP* *Gallbladder* *Chromatography* *Pharmacology*

1. INTRODUCTION

Peptide histidine isoleucine (PHI) is a 27 amino acid peptide isolated from porcine gut which shows considerable amino acid sequence similarities to members of the glucagon-secretin family of peptides [1]. PHI immunoreactive (PHI-IR) material has been demonstrated in both gut [2,3], and brain [4] suggesting a possible physiological role as a neurotransmitter or neuromodulator. Indeed, PHI has recently also been purified from porcine brain and shown to be identical to the intestinal peptide [5].

PHI has a number of biological activities in common with VIP. Both peptides are potent stimulants of pancreatic secretion [6–9] and intestinal secretion in several species [10–12]. In addition it has been shown that PHI can displace VIP label bound to membrane receptors from lung [13]. However, unlike VIP, PHI is only a weak vasodilator [14].

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Authors in [15] have recently reported the existence of a number of regulatory peptides, present in nerves and mucosal endocrine cells of the guinea pig gall bladder. PHI has been shown to reduce basal bilirubin and bile acid output in man [16] and to decrease basal gallbladder pressure and fluid reabsorption in the guinea pig [17]. PHI may thus be an additional local peptide involved in gallbladder function. We have, therefore, investigated the distribution of PHI-IR in the guinea pig gallbladder by radioimmunoassay and immunocytochemistry and examined the effect of PHI on a gallbladder smooth muscle preparation in vitro.

2. MATERIALS AND METHODS

For radioimmunoassay, the gallbladder, the common duct and the cystic duct of 7 Dunkin–Hartley guinea pigs were obtained after the animals were killed by cervical dislocation. The tissues were immediately cleaned with water, weighed and rapidly placed into polypropylene

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00145793/84/$3.00

tubes containing boiling 0.5 mol/l acetic acid (~10 ml/g of tissue). Aliquots (10 μl) of the supernatant were measured in duplicate for PHI immunoreactivity.

2.1. *Radioimmunoassay*

A full description of the assay has previously been reported [2]. The PHI antiserum was produced following immunisation with porcine PHI conjugated to bovine serum albumin (BSA) with glutaraldehyde. The antibody used in this study (designated T41) did not cross-react with up to 100 pmol/tube of all available peptides including the structurally related VIP, GIP, glucagon, glicentin, secretin, and human pancreatic growth hormone releasing factor (hpGRF). Iodination of PHI was performed using the Iodogen method and purified from unreacted PHI and ^{125}I by reverse-phase high pressure liquid chromatography (HPLC) using isocratic conditions: column: μ-Bondapak (Waters Associate); mobile phase: 32% acetonitrile containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 ml/min. The assay was carried out in phosphate buffer containing 0.01% of detergent (Tween 20), 0.3% bovine serum albumin (BSA) and 1% aprotinin.

'Antibody-bound label' and 'free label' were separated by dextran-coated charcoal (4 mg/tube) after 5–7 days incubation at 4°C. The inter-assay and intra-assay variations were 13 and 5% respectively. The assay could detect changes of 0.5 fmol/tube with 95% confidence.

2.2. *Chromatography*

Gallbladder extracts were examined by gel permeation and HPLC. For gel permeation analysis, samples were applied to a column (0.9 × 60 cm) of Sephadex G-50 superfine (Pharmacia) and eluted at a flow rate of 3.6 ml/h at 4°C with phosphate buffer, pH 7.2 containing 0.3% BSA and 0.02% Tween 20 (Sigma). The columns were precalibrated with dextran blue (M_r 2 × 10^6), horse heart cytochrome *c* (M_r 12384) and a trace amount of Na^{125}I, which were also added to samples in trace amounts as internal markers. In addition, porcine PHI standard was chromatographed separately so that a comparison of elution position could be made. The partition coefficient (K_{av}) between the available stationary phase and mobile phase was calculated according to [18].

Tissue extracts were also applied onto a Waters HPLC system using a pre-packed μ-Bondapak C_{18} column (3.9 × 300 mm). Samples were prepared as previously described [22]. PHI-IR was eluted using a gradient of 25–50% acetonitrile/0.1% TFA. The flow rate was 1 ml/min with 1-ml fractions collected for subsequent radioimmunoassay.

2.3. *Immunocytochemistry*

For immunocytochemistry, the gallbladder, the common bile duct and the cystic duct were removed and fixed immediately in 0.4% *p*-benzoquinone (BQS) for 1 h at room temperature [19]. After washing overnight in phosphate-buffered saline (PBS) containing 20% sucrose, the tissues were made into cryostat blocks by freezing in Arcton. 10-μm thick cryostat sections were cut. The sections were picked up on poly(L-lysine) (PLL)-coated glass slides [20] and allowed to dry for 1 h at room temperature. Before immunostaining the sections were soaked in PBS containing 0.2% Triton X-100. After washing briefly in PBS, a modified indirect immunofluorescence method [21] was applied, in which the first layer antiserum was repeated. The antiserum to PHI was diluted 1:500 in PBS. The controls included preabsorption of the first layer antiserum with natural and synthetic PHI, the omission of the first layer and the use of normal rabbit serum as the first layer.

2.4. *Pharmacology*

Six Dunkin-Hartley guinea pigs were killed by cervical dislocation, and the gallbladders were removed, opened out and cut into single zig-zag strips [23]. The tissues were arranged in cascades [24–26] and superfused at 5 ml/min with a Tyrode solution bubbled with 95% O_2, 5% CO_2 at 37°C. The sensitivities of the preparations to noradrenaline were first demonstrated, and increasing amounts of PHI and VIP (2–200 pmol) were then tested. A mixture of antagonists to catecholamines (phenoxybenzamine hydrochloride 10^{-7} g/ml and propranolol hydrochloride 2 × 10^{-6} g/ml), histamine (mepyramine maleate 10^{-7} g/ml), 5-hydroxytryptamine (methysergide bimaleate 2 × 10^{-7} g/ml) and acetylcholine (hyoscine hydrochloride 10^{-7} g/ml) was also tested. A tension of 1 g was applied to the strips and the responses to the peptides were recorded isometrically with force transducers (Grass FT03C)

Table 1

Regional distribution of PHI-IR in the guinea pig gallbladder

	PHI-IR pmol/g (wet wt of tissue)
Gallbladder	6.3 ± 0.6
Common duct	5.0 ± 0.7
Cystic duct	7.8 ± 1.9

on a Grass polygraph (Model no. 70). Experiments were started after a 60-min equilibration period.

3. RESULTS

Table 1 shows the levels of PHI-IR (pmol/g wet wt of tissue and quoted as the mean ± standard error of mean (SE)) in the gallbladder, common and cystic duct. PHI-IR was present with similar levels (range: 5.0–7.8 pmol/g) in all three areas examined.

Examination of gallbladder extracts by gel permeation (fig.1) shows that they contain a PHI-like peptide which co-elutes with porcine PHI (K_{av} 0.53). Following HPLC of gallbladder extracts, only one major form of PHI-IR (fig.2) was eluted.

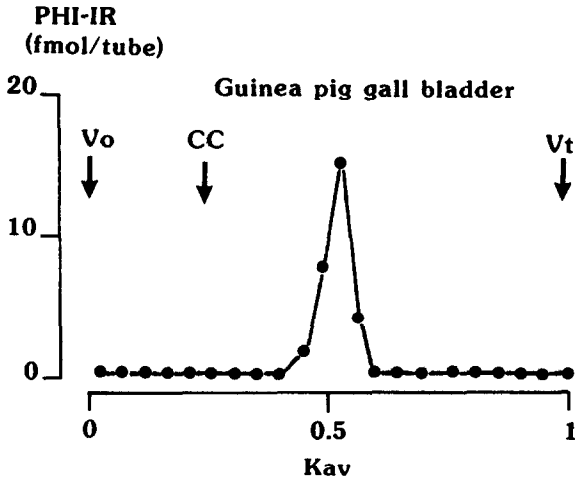

Fig.1. Representative gel chromatographic profile of guinea pig gallbladder acid extracts on Sephadex G-50 superfine. Arrows indicate position of dextran blue (V_o), cytochrome *c* (CC) and $Na^{125}I$ (V_t). A similar chromatographic profile was observed with the pure porcine PHI standard.

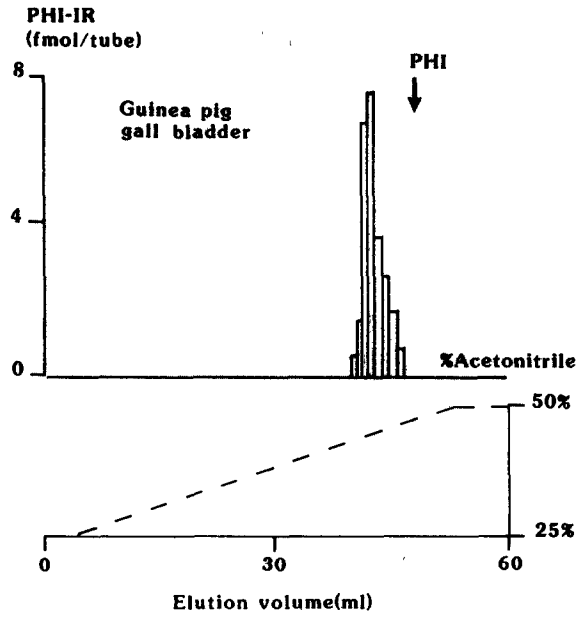

Fig.2. Fractionation of guinea pig gallbladder acid extracts on a Waters reverse-phase HPLC system [22], using a linear gradient of 25 to 50% acetonitrile. The guinea pig PHI immunoreactivity eluted at 42 ml and the porcine PHI standard (arrowed) at 47 ml.

This PHI-IR was eluted significantly earlier than the porcine PHI standard. Recoveries for all chromatographic runs were 93 ± 7%.

PHI-immunoreactivity was localised to nerves which were distributed throughout the tissue examined. In the gallbladder such nerves were more abundant in the ganglionic plexus than in other

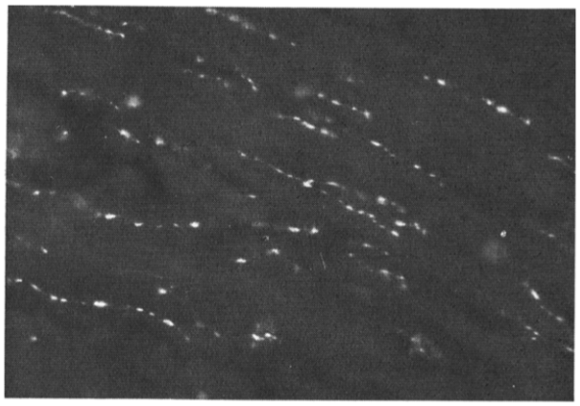

Fig.3. Varicose PHM-IR nerve fibres in the smooth muscle layer of the gallbladder of guinea pig (× 332).

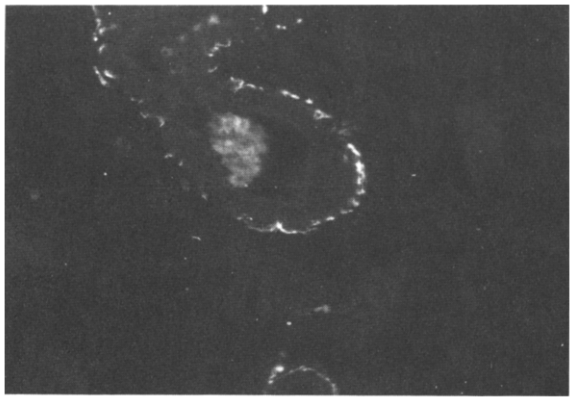

Fig.4. PHM-immunoreactive nerve fibres surrounding an arteriole in guinea pig gallbladder wall (× 358).

regions (fig.3). The PHI-IR nerves formed networks with numerous varicosities and ran in the connecting strands between different groups of ganglion cells. Such nerve fibres sometimes surrounded ganglion cells, but the ganglion cells themselves were not convincingly observed to contain PHI-immunoreactivity. The muscle layer of the gallbladder was innervated by PHI-IR nerves. In the cystic and common bile ducts, the PHI-immunoreactivity was mainly distributed in the muscle layers (fig.4). The frequency of these nerves in the muscle layer of the common bile duct gradually increased toward the sphincter. In general, the distribution pattern of PHI-IR nerves was very similar to that of VIP-IR ones reported previously [15], but the population of the former was considerably smaller. All the controls gave negative results.

Porcine PHI induced a dose-dependent relaxation of the guinea pig gallbladder which was not blocked by any of the inhibitors tested. The onset of action was immediate and maxima were achieved within 10 min. A comparison of the basal relaxant effects of PHI and VIP showed parallel concentration-effect responses (fig.5).

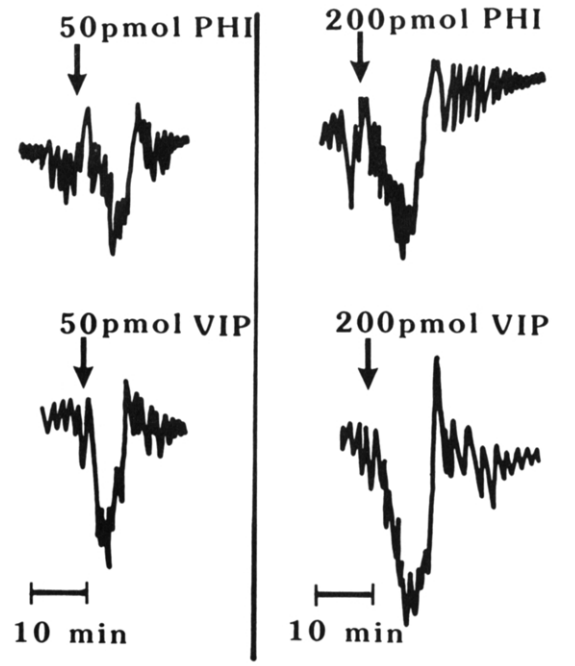

Fig.5. Responses of isolated, superfused guinea pig gallbladder preparations to two doses (50 and 200 pmol) of PHI and VIP. All agents were dissolved in Tyrode solution. Peptide solutions were applied as indicated by arrows. Upward movement is contraction and downward movement relaxation.

4. DISCUSSION

Our results show that guinea pig gallbladder contains a significant quantity of PHI-IR which has been localised by immunocytochemistry to ganglionated and mucosal plexus of the gallbladder. These immunoreactive ganglion cells have a similar distribution to those of VIP-containing cells, indeed, in the gut the ganglionic cells appear to contain both VIP and PHI [3]. In addition, concentrations of PHI-IR quoted here are very similar to those reported for VIP-IR [15]. These results, therefore, are in agreement with the concept of a common precursor for VIP and PHI [27]. Indeed, a precursor has recently been identified in a human neuroblastoma cell line [28] and in a single vipoma [29], which contains the entire sequence of VIP and a PHI-like peptide differing by only 2 amino acid residues (Arg^{12} replaced by Lys and Ile^{27} by Met).

Gel permeation analysis of gallbladder extracts suggested that PHI-IR in the guinea pig is of a similar size as the porcine PHI standard. However, since the guinea pig PHI-IR was eluted at a different position to porcine PHI following analysis by HPLC, it appears that the two sequences vary slightly.

The gallbladder is innervated by excitatory cholinergic nerves and inhibitory smooth-muscle

relaxant adrenergic nerves [30–34]. The existence of additional neural control factors was demonstrated by the failure to inhibit responses of the gallbladder to electrical field stimulation [35–37], by adrenergic or cholinergic blockade. The presence of biologically active peptides in the gallbladder [15,38] suggested that these peptides may serve to mediate this non-adrenergic, non-cholinergic inhibitory response. Some peptides have been excluded because they do not cause relaxation of the gallbladder, e.g., bombesin [39] and CCK-8 [40]. The possibility that VIP may be a mediator was suggested by several observations including its presence in the gallbladder [15,41], and its ability to relax gallbladder smooth muscle independently of adrenergic or cholinergic receptors [42].

A possible physiological action of PHI in the gallbladder was first suggested by authors in [17] who reported that PHI is capable of decreasing basal gallbladder pressure, and that PHI reduces bilirubin and bile acid output in man [16]. The results here, showing that PHI-IR is present in nerves of the gallbladder and that it possesses the ability to cause a relaxation of the guinea pig gallbladder muscle which is not blocked by many antagonists, suggests that PHI may also have a physiological role in gallbladder function.

ACKNOWLEDGEMENTS

This study was supported by grants from the Medical Research Council and the British Diabetic Association.

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